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The Effect of TYB-2285 on Dual Phase Bronchoconstriction and Airway Hypersensitivity in Guinea-pigs Actively Sensitized with Ovalbumin

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Abstract—The effect of a new anti-asthmatic drug, TYB-2285 (3,5-bis(acetoxyacetylamino)-4-chlorobenzonitrile), was investigated in ovalbumin-sensitized guinea-pigs. When guinea-pigs were pretreated with TYB-2285 (300 mg kg^{-1}, p.o., single dose or consecutively for 7 days), the immediate asthmatic response was inhibited as demonstrated by diminished cyanosis, but not the bronchoconstriction. TYB-2285, given singly or consecutively, inhibited the appearance of late asthmatic response and the infiltration of inflammatory cells, such as eosinophils, into the airway. Additionally, airway hyper-responsiveness was also reversed by the single administration of TYB-2285. Luminol-dependent chemiluminescence of airway-infiltrated cells stimulated with A23187 was inhibited by TYB-2285 in a dose-dependent manner. The present study suggests that TYB-2285 inhibits late asthmatic response and airway hyper-responsiveness by inhibiting the accumulation of eosinophils and other inflammatory cells into the airway, and also by inhibiting the production of oxygen radicals from airway-infiltrated cells.

TYB-2285 (3,5-bis(acetoxyacetylamino)-4-chlorobenzonitrile) is a new anti-asthmatic compound which possesses an anti-allergic action (Watanabe et al 1989) and an anti-inflammatory action (Watanabe et al 1992). Cortes et al (1992) demonstrated that TYB-2285 suppressed airway hyper-responsiveness in naturally sensitized sheep. This compound inhibited T-cell-dependent influx of eosinophils in actively-sensitized rats (Tominaga et al 1992). Our previous study (Tohda 1989) demonstrated that the inhalation of a low concentration of specific antigen to actively-sensitized guinea-pigs induced immediate and late asthmatic responses. The aim of the present study was to investigate the effect of TYB-2285 on asthmatic symptoms in actively-sensitized guinea-pigs. Additionally, the effects of TYB-2285 on the accumulation of inflammatory cells into the airway and on luminol-dependent chemiluminescence of airway-infiltrated cells stimulated with A23187 were investigated.

Materials and Methods

Animals

Dunkin Hartley strain male guinea-pigs, 250–350 g, were used.

Reagents

Cyclophosphamide (Shionogi, Osaka, Japan), ovalbumin (Grade V, Sigma, St Louis, USA), $Al(OH)_3$ (Wako Pure Chemical, Osaka, Japan), histamine dihydrochloride (Wako Pure Chemical), trypan blue (Wako Pure Chemical), pentobarbitone sodium (Abbott Laboratories, North Chicago, USA), luminol solution (Labo-Science, Tokyo, Japan), Hank's balanced salt solution (HBSS, Whittaker Bioproducts, Walkersville, USA), calcium ionophore A23187 (A23187, Sigma), carmellose sodium salt (CMC-Na, Nakarai Tesque, Kyoto, Japan) and dimethylsulphoxide (DMSO, Merck, Darmstadt, Germany) were used. TYB-2285 was synthesized at the Pharmaceuticals Research Center of Toyobo Co. Ltd. For in-vivo study, TYB-2285 was suspended in 0·5% CMC-Na. For in-vitro study, TYB-2285 was dissolved in DMSO (final concentration of DMSO; 0·5%).

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Sensitization

Guinea-pigs were actively sensitized with ovalbumin according to the method described previously (Tohda 1989). Briefly, guinea-pigs were sensitized intraperitoneally with the mixture of 1 mg ovalbumin and 100 mg $Al(OH)_3$ two days after the intraperitoneal injection of cyclophosphamide (30 mg kg^{-1}). Animals were boosted by the intraperitoneal injection of a mixture of 10 μg ovalbumin and 100 mg $Al(OH)_3$ three weeks after the first sensitization. Animals were used for experiments three weeks after the booster injection.

Antigen provocation

Guinea-pigs inhaled aerosolized ovalbumin (2 mg mL^{-1}) generated by a nebulizer (DeVilbiss 646 type) for 1 min.

Drug administration

In the control group, sensitized animals were given orally 0·5% CMC-Na solution (4 mL kg^{-1}). For the TYB-2285 (300 mg kg^{-1}) single-dosed group, TYB-2285 was given orally 15 min before the antigen provocation, or 15 min before histamine inhalation. In the TYB-2285 (300 mg kg^{-1} day^{-1}) multi-dosed group, TYB-2285 was given orally consecutively for seven days, before antigen provocation.

Analysis of pulmonary function

Our previous study (Tohda 1989) demonstrated a good relationship among tidal volume (V_T), lung resistance, dynamic compliance, respiratory frequency and peak expiratory flow rate. Therefore, to minimize stress on

guinea-pigs, V_T was measured as a parameter of asthmatic response. When V_T decreased more than 20% from baseline, asthma-like bronchial response was considered to be positive. Immediate asthmatic response was defined as the presence of this type of bronchial response immediately after the antigen inhalation. Late asthmatic response was defined as reappearance of this type of bronchial response within a few hours after the immediate asthmatic response had disappeared.

Bronchoalveolar lavage (BAL)

BAL was performed before the antigen provocation, 10 min and 4 h after the antigen provocation. After guinea-pigs were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.p.), they were intubated with a sterile endotracheal tube. BAL was performed with saline (5 mL kg^{-1} × 4). The BAL fluid was immediately placed in sterile tubes on ice. Total living cells were counted with an improved Neubauer haematocytometer in the presence of trypan blue. Slides were made from cytospin (200 *g*, 5 min) and stained with May–Grünwald–Giemsa's stain to determine cellular components.

Assessment of airway reactivity to histamine

Peak P_{ao} (airway opening pressure) was measured 1 min after the inhalation of various concentrations of histamine. Sensitized or non-sensitized guinea-pigs were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.p.). After tracheotomy, the trachea was cannulated (i.d. 2·0 mm) and ventilated with a mechanical respirator (tidal air flow, 10 mL kg^{-1}, 60 strokes min^{-1}, Model 680, Harvard). Change in inspiratory P_{ao} was measured by a differential pressure transducer (TP-603T, Nihon Kohden) through a lateral branch of the tube connected to the tracheo-cannula. An inhalation apparatus for inducing the airway responsiveness to histamine was constructed by connecting an ultrasonic nebulizer (NE-10, Omron) to a ventilation system (Minami et al 1983). Histamine at concentrations of 4·9, 9·8, 20, 39, 78 or 156 $\mu g\,mL^{-1}$ was inhaled for 30 s every 5 min.

Luminol-dependent chemiluminescence (LDCL) of BAL cells

BAL was performed in ovalbumin-sensitized guinea-pigs as described above and then airway-infiltrated cells were resuspended in HBSS at a concentration of 10^6 cells mL^{-1} and were used as BAL cells. TYB-2285 solution was prepared at a concentration of 5, 10 or 20 $\mu g\,mL^{-1}$. After

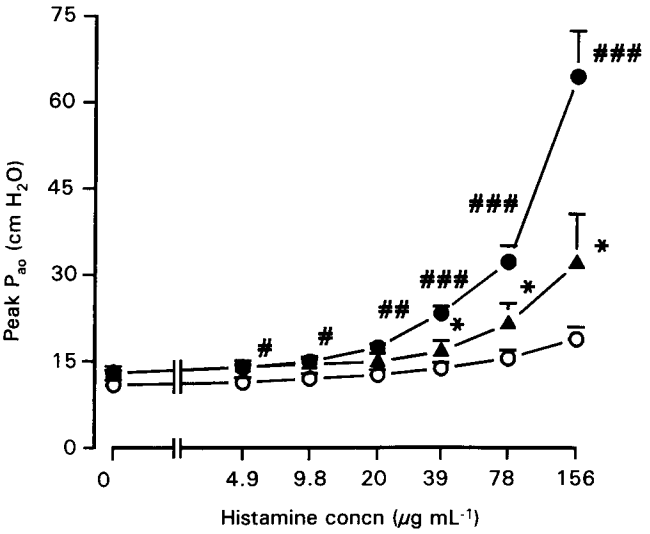

FIG. 1. Effect of TYB-2285 (300 mg kg^{-1}) on airway hyper-responsiveness to histamine induced by antigen sensitization. $^{\#}P < 0{\cdot}05$, $^{\#\#}P < 0{\cdot}01$, $^{\#\#\#}P < 0{\cdot}001$ vs non-sensitized animals, respectively. $^{*}P < 0{\cdot}05$ vs control sensitized animals. ○ Non-sensitized, ● sensitized, ▲ sensitized + TYB-2285.

80 μL BAL cells was incubated with 20 μL TYB-2285 or control solution (0·5% DMSO) at 37 °C for 10 min, 50 μL 2×10^{-4} M luminol solution was added. BAL cells were stimulated with 25 $\mu g\,mL^{-1}$ A23187. The production of oxygen radicals by BAL cells stimulated with A23187 was measured by the LDCL method using a lumiphotometer (TD-4000, Labo-Science). The peak values and integral values of LDCL were calculated by measuring LDCL at 37 °C for 10 min.

Statistical analysis

Data were analysed using a two-tailed unpaired or paired Student's *t*-test within groups. $P < 0{\cdot}05$ was considered significant. Variance was expressed as the standard error of the mean.

Results and Discussion

The results of the study are summarized in Tables 1–3 and in Fig. 1. TYB-2285 inhibited the late asthmatic response (Table 1). There were marked and statistically significant increases in total cells, eosinophils and neutrophils at the

Table 1. Effect of TYB-2285 on immediate and late asthmatic responses in actively-sensitized guinea-pigs.

Group	n	Immediate		Late
		Bronchoconstriction (%)	Cyanosis (%)	Bronchoconstriction (%)
Control	5	100·0	100·0	100·0
TYB-2285 300 mg kg^{-1}	12	91·7	75·0	25·0
TYB-2285 300 mg kg^{-1} day^{-1} for 7 days	6	100·0	33·0	16·7

Results are shown as appearance (%) of bronchoconstriction or cyanosis.

Table 2. Effect of TYB-2285 on BAL components.

	Total cells (cells mm^{-3})	Eosinophils (cells mm^{-3})	Neutrophils (cells mm^{-3})	Macrophages (cells mm^{-3})	Lymphocytes (cells mm^{-3})
Baseline					
Group 1	440·0 ± 50·1	51·5 ± 13·7	9·7 ± 3·7	369·9 ± 37·6	8·9 ± 2·7
Group 2	631·7 ± 104·8	83·6 ± 19·1	5·9 ± 3·2	509·7 ± 90·0	32·5 ± 11·1*
Group 3	422·5 ± 72·9	42·3 ± 16·7	8·2 ± 2·6	366·3 ± 56·6	5·6 ± 1·0
Intermediate asthmatic response					
Group 1	347·5 ± 48·2	43·1 ± 9·5	9·8 ± 2·8	287·0 ± 42·5	8·0 ± 2·4
Group 2	237·5 ± 42·1	52·8 ± 19·2	2·2 ± 0·9*	117·3 ± 24·5	5·2 ± 2·2
Group 3	510·3 ± 37·0*	98·6 ± 7·0**	12·8 ± 1·6	378·6 ± 31·6	20·2 ± 8·9
Late asthmatic response					
Group 1	1368·6 ± 177·5##	600·0 ± 90·1###	83·4 ± 19·5#	676·5 ± 76·8#	10·0 ± 3·7
Group 2	591·7 ± 107·6**	153·2 ± 32·5**	13·0 ± 4·4**	401·8 ± 81·0*	23·7 ± 4·8
Group 3	729·3 ± 117·8*	156·7 ± 32·8**	23·3 ± 6·3*	511·4 ± 76·1*	37·9 ± 10·0*

Results are expressed as mean ± s.e.m. of 3–7 animals. Baseline: before the antigen provocation; immediate asthmatic response: 10 min after the antigen provocation; late asthmatic response: 4 h after the antigen provocation; group 1: control group; group 2: TYB-2285 (300 mg kg^{-1}) single-dosed group; group 3: TYB-2285 (300 mg kg^{-1}) multi-dosed group. *$P < 0.05$, **$P < 0.01$ vs control group, #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs control group of baseline.

late phase (4 h after the antigen provocation), but not at the immediate phase (10 min after the antigen provocation) (Table 2). TYB-2285 inhibited the increase in cell numbers at the late phase. TYB-2285 partially inhibited LDCL of BAL cells stimulated with A23187 (Table 3). Fig. 1 shows that the airway reactivity is increased by antigen sensitization. Single administration of TYB-2285 reversed this increase in sensitized animals.

Inhalation of specific antigens to allergic asthma patients provokes two different types of asthmatic attack, immediate and late asthmatic responses (Robertson et al 1974). Immediate asthmatic response is based on a type I allergic reaction, but the mechanism of late asthmatic response is not yet clear. Late asthmatic response shares common properties with severe chronic asthma, including accompanying airway hyper-responsiveness (Cockcroft et al 1977), infiltration of eosinophils into bronchial tissues (Frigas et al 1981), poor responsiveness to β-stimulants (Robertson et al 1974) and desquamation of airway epithelial cells (Frigas & Gleich 1986). It has been suggested that eosinophils (Frigas et al 1981), neutrophils (Nagy et al 1982) and other inflammatory cells might play important roles in the pathogenesis of late asthmatic response. This is supported by several animal experiments (Murphy et al 1986; Iijima et al 1987; Fukuda et al 1990), and it is considered important to inhibit the infiltration of inflammatory cells such as eosinophils into the airway and to inhibit the function of inflammatory cells in the management of asthma.

The present study suggests that TYB-2285 might inhibit the late response by inhibiting the accumulation of inflammatory cells into the airway. Robertson et al (1974) speculated that the late response following an immediate response is a continuation of type I allergic reaction. However, the finding that the pre-treatment of TYB-2285 did not affect the occurrence of the immediate response and only inhibited cyanosis during this response suggests that TYB-2285 inhibits the late response independently of the inhibition of the immediate response, and that the late response may not be a simple continuation. The evidence that TYB-2285 inhibited carrageenan-, zymosan-, bromelain- or Arthus-induced paw oedema and zymosan-induced pleurisy (Watanabe et al 1992) might also support our speculation. However, it cannot be ruled out completely that TYB-2285 inhibits late asthmatic response by inhibiting the release of mediators which induce late response, since TYB-2285 remarkably inhibited anaphylactic release of histamine and thromboxane A_2 in rats (Watanabe et al 1989, 1991).

Among mediators produced from inflammatory cells, oxygen radicals (Cluzel et al 1987) are thought to play an important role in the inflammatory process in the bronchus. Eosinophils stimulated with leukotriene B_4 or platelet-activating factor generate active oxygen, causing tissue damage (Tauber et al 1979; Palmblad et al 1984; Kloprogge et al 1989). Uenishi (1990) reported that LDCL of BAL cells increased in ovalbumin-sensitized guinea-pigs compared with that in non-sensitized guinea-pigs. He also reported that LDCL increased relative to the amount of sensitizing antigen. Uenishi suggested that active oxygen was involved in late asthmatic response by priming eosinophils. LDCL of BAL cells was inhibited at a concentration of 20 μg mL^{-1} TYB-2285. This result suggests that TYB-2285 inhibits late

Table 3. Effect of TYB-2285 on luminol-dependent chemiluminescence of BAL cells.

	Concentration of TYB-2285 (μg mL^{-1})			
	0	5	10	20
Peak value	1·58 ± 0·45	1·65 ± 0·22	1·46 ± 0·31	0·97 ± 0·29
Integral value	684·7 ± 168·0	785·3 ± 68·0	666·0 ± 119·2	439·7 ± 137·8*

Results are expressed as mean ± s.e.m. of three experiments. *$P < 0.05$ vs 0 μg mL^{-1} TYB-2285.

asthmatic response, not only by inhibiting the accumulation of inflammatory cells into the airway, but also by suppressing the activation of inflammatory cells.

The airway smooth muscle of asthma patients (Cockcroft et al 1977) is very sensitive to bronchoconstrictors such as histamine, methacholine and acetylcholine, and bronchial inflammation (Barnes 1986) is heavily involved in the acceleration of bronchial hypersensitivity. It has been reported that macrophages from asthma patients release more active oxygen than those from healthy individuals (Cluzel et al 1987). Active oxygen may be involved in the pathogenesis of bronchial hypersensitivity. Since TYB-2285 does not have a direct anti-histamine effect (Watanabe et al 1989), TYB-2285 may be considered to inhibit bronchial hypersensitivity to histamine by suppressing the production of active oxygen and some other mediators from BAL cells primed by active sensitization, supported by evidence that TYB-2285 inhibits histamine release from primed mast cells (Watanabe et al 1993) and primed basophils (Tominaga et al 1993).

Our data support the notion that late asthmatic response has common features with the pathogenesis of chronic refractory asthma such as airway hyper-responsiveness and bronchial inflammatory cell infiltration.

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